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Original research article

Prazosin-stimulated release of hepatic triacylglyceride lipase from primary cultured rat hepatocytes is involved in the regulation of cAMP-dependent protein kinase through activation of the Ca^{2+}/calmodulin-dependent protein kinase-II

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ABSTRACT

Background: Prazosin is an α1 adrenoceptor antagonist used in pharmacotherapy for the treatment of hypertension. Prazosin alters lipid metabolism *in vivo*, but the involved mechanism is not fully understood. In this study, we investigated the mechanism underlying the alteration of lipid metabolism. We show that the prazosin-stimulated release of hepatic triacylglyceride lipase (HTGL) from primary cultured rat hepatocytes involved Ca^{2+}/calmodulin-dependent protein kinase II (CaMK-II) activation.
Methods: Primary cultured rat hepatocytes were incubated with prazosin and other agents. The hepatocytes were used in the CaMK-II and protein kinase A (PKA) activity assay. The supernatant was used in the HTGL activity assay and western blotting.
Results: Prazosin-stimulated HTGL release was suppressed by the inositol triphosphate receptor inhibitor xestospongin C and by the calmodulin inhibitor trifluoperazine but not by the protein kinase C inhibitor chelerythrine chloride or a diacylglycerol kinase inhibitor (R59949). Furthermore, the calmodulin-dependent protein kinase II (CaMK-II) activity in prazosin-treated hepatocytes increased in a time- and dose-dependent manner. The cAMP-dependent PKA activity of prazosin-stimulated hepatocytes was suppressed by a phospholipase C (PLC) inhibitor (U-73122), trifluoperazine, and a CaMK-II inhibitor (KN-93).
Conclusions: These results suggested that prazosin-stimulated HTGL release from hepatocytes was caused by activation of PKA associated with stimulation of CaMK-II activity through a signal cascade from PLC.

Introduction

Hepatic triacylglyceride lipase [(HTGL), EC 3.1.1.3] hydrolyzes triacylglycerides in intermediate- and high-density lipoproteins (HDLs) and is considered to have an important role in lipid metabolism [1–3]. HTGL deficiency, which causes xanthoma and coronary heart disease, develops from a large TG-rich HDL accumulation and very low-density lipoprotein (LDL) remnants. This enzyme is synthesized and glycosylated in the cytosolic fraction of hepatocytes, extracellularly secreted, and released onto the cell surface and vascular endothelium [4]. The secreted enzyme is anchored to the surface and to vascular endothelial cells by electrostatic binding with heparan sulfate proteoglycans [5]. However, the transport process and regulation of HTGL release from hepatocytes remain unclear.

Prazosin [1-(4-amino-6,7-dimethoxy-quinazolin-2-yl)-4-(2-furoyl)piperazine] is generally used for treating spontaneous renal hypertension and prostatic hyperplasia with dysuria; it does so by blocking the α1 adrenoceptor [6,7]. In addition, an antihypertensive with a quinazoline structure containing prazosin is also reportedly involved in lipid metabolism, with actions such as increasing HDL cholesterol levels and the cholesterol ratio in hypertensive patients by long-term administration [8]. In animal experiments, prazosin has been reported to decrease 3-hydroxy-3-methylglutaryl-CoA reductase activity in mouse serum [9], increase lipoprotein lipase activity in adipose tissue [10], and express upregulation of LDL receptor in high-cholesterol-diet-fed Japanese monkeys [11]. This evidence suggests that prazosin has

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some role in lipid metabolism. In addition, we recently reported on the prazosin-stimulated release of HTGL from primary cultured rat hepatocytes [12]. The results showed that the release of HTGL from these hepatocytes was promoted by prazosin but not by other α1 adrenoceptor blockers, such as doxazosin. Prazosin-stimulated release of HTGL appeared to be adrenoceptor independent and provided important signaling through the activation of phospholipase C (PLC). This HTGL release appeared to cause an increase in intracellular Ca^{2+} and calmodulin-dependent protein kinase II. Furthermore, HTGL release also appeared to activate cAMP-dependent protein kinase A (PKA) that was induced by cAMP in hepatocytes [13]. This finding showed that HTGL release was involved in the activation of PKA accompanied by prazosin-stimulated increases in intracellular cAMP through adenylate cyclase. However, the details of the signal cascade mechanism, including the interactions among the signaling molecules, remain unclear.

Thus, we investigated whether prazosin-stimulated HTGL release from rat hepatocytes was caused by activation of CaMK-II in these cells, and we studied the associated signaling interactions.

Materials and methods

Materials

ATP, [γ-^{32}P] – (111 TBq/mmol), and triolein, [carboxyl-^{14}C] – (2.59 GBq/mmol), were purchased from Perkin Elmer (Yokohama, Japan). Prazosin, collagenase, trifluoperadine, and xestospongin C were obtained from Wako Pure Chemical Industries (Osaka, Japan). R59949, Phorbol 12-myristate 13-acetate (TPA), and Williams' medium E were purchased from Sigma (St. Louis, MO, USA). The Signa TECT® CaMK-II assay system (V8161) was manufactured by Promega (Madison, WI, USA). Chelerythrine chloride and PKA assay kits (# 17-134) were obtained from Merck Millipore (Billerica, MA, USA). All other chemicals used in the study were of analytical grade.

Animals

Male Wistar rats (weight, 200–300 g) were purchased from Shimizu Laboratory Animal Supplies (Kyoto, Japan), were fed commercial laboratory chow *ad libitum*, and were fasted for 24 h before the experiments. The animals were cared for according to the "Guide for the Care and Use of Laboratory Animals" established by Fukuyama University.

Hepatocyte preparation and culture

Hepatocytes were isolated by *in vitro* collagenase perfusion and low-speed centrifugation with modifications. Kupffer cell contamination in the hepatocyte preparations was confirmed to be <2% by peroxidase staining [14]. Cell viability was determined by trypan blue exclusion and ranged from 85 to 95%. The isolated hepatocytes were cultured for 24 h as monolayers in a plastic dish (1×10^5 cells/cm^2) containing Williams' medium E supplemented with 10% fetal calf serum, 10-nM insulin, 10-nM dexamethasone, and 5-kIU/mL aprotinin and were incubated in a 5% CO_2 atmosphere. After removing the medium by aspiration, the hepatocyte monolayers were incubated for an additional 0–60 min in Williams' medium E supplemented with 2% bovine serum albumin and prazosin and other agents. Hepatocytes were harvested, centrifuged at $50 \times g$ for 10 min to remove cellular debris, and then used in the CaMK-II and PKA activity assay. The supernatant was used in the HTGL activity assay.

HTGL activity determination

HTGL activity was determined using triolein [carboxyl-^{14}C] – (3.7 MBq/mL) as the substrate [15]. HTGL activity was expressed as pmol of free fatty acids produced/min/10^6 cells.

Western blotting

Antibodies against HTGL were purchased from LabFrontier (Seodaemun-gu, Seoul, Korea), and the horseradish peroxidase (HRP)-conjugated secondary anti-rabbit antibody was purchased from Bio-Rad (Hercules, CA, USA). Protein estimation was carried out using the Bradford method. Approximately 15 μg of protein from the cell supernatant was mixed in a sample buffer, boiled for 5 min at 95 °C, loaded onto each lane containing 10% gel, and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and then the resolved proteins were electrotransferred to polyvinylidene fluoride membranes (GE Healthcare UK Ltd., Buckinghamshire, England). The membranes were blocked with 1% non-fat dry milk in phosphate-buffered saline (PBS) with 0.1% Tween 20 for 1 h at room temperature. After blocking, the membrane was washed five times with PBS for 5 min and then incubated with the primary antibody (1:2000 dilution) for 1 h at room temperature. It was then washed five times with PBS for 5 min and incubated with an HRP-conjugated secondary antibody (1:5000 dilution) for 1 h at room temperature. After washing with PBS, the bands were detected by an enhanced chemiluminescence substrate (Wako Pure Chemical Industries, Osaka, Japan).

CaMK-II activity determination

Hepatocytes incubated with prazosin were homogenized in 20-mM Tris–HCl (pH 8.0) containing 2-mM ethylenediamine tetraacetic acid, 2-mM ethylene glycol tetraacetic acid, 20-μg/mL soybean trypsin inhibitor, 10-μg/mL aprotinin, 5-μg/mL leupeptin, 2-mM dithiothreitol, 25-mM benzamidine, and 1-mM phenylmethylsulfonyl fluoride using a Vibra-cell ultrasonic processor (model VC-130PB; SONICS, CT, USA) and then centrifuged at $350 \times g$ for 5 min at 4 °C. The supernatant was used as the enzyme solution to determine CaMK-II activity along with a Signa TECT® CaM KII assay system (V8161). The biotinylated peptide substrate was indirectly quantified through the phosphorylated ATP, [γ-32P]- (111 TBq/mmol), absorbance. Radioactivity was measured and expressed as nmol phosphate/min/10^6 cells.

PKA activity determination

Hepatocytes pre-incubated for 10 min with several inhibitors and incubated for 60 min with or without prazosin were homogenized in 20-mM MOPS buffer (pH 7.2), which contained 25-mM β-glycerophosphate, 5-mM ethylene glycol tetraacetic acid (EGTA), 1-mM sodium orthovanadate, and 1-mM dithiothreitol, using a Vibra-cell ultrasonic processor (model VC-130PB; SONICS), and then centrifuged at $10{,}000 \times g$ at 4 °C for 20 min. The supernatant was used as an enzyme preparation to determine PKA activity along with a PKA assay kit. Radioactivity was measured and expressed as pmol phosphate/min/10^6 cells.

Data analysis

Results are expressed as the means ± standard error (SE) of three or four determinations from independent experiments using different hepatocyte preparations. The data were analyzed by performing an unpaired Student's *t*-test and Dunnett's test.